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Cancer Therapeutics in Drosophila melanogaster: A Closer Look at the ErbB Family of Receptor Tyrosine Kinases

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Cancer Therapeutics in *Drosophila melanogaster*: A Closer Look at the ErbB Family of Receptor Tyrosine Kinases

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ABSTRACT

The Epidermal Growth Factor Receptor (EGFR/ErbB) receptor tyrosine kinase (RTK) family has been implicated in numerous cancer types (e.g. lung, breast and brain) and this signaling network is conserved from *Drosophila* to humans. For this reason, *Drosophila* may provide an ideal in vivo system for experimental analyses of this pathway and a chemical genetics approach to the identification of therapeutics against it. Towards this goal, an assay utilizing *Drosophila* transgenic for members of the human ErbB family was developed for screening putative inhibitory compounds. Preliminary phenotypic data with known EGFR inhibitors yielded promising results.

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BACKGROUND

Cancer is a worldwide problem, affecting millions of people from every age, gender, race, and nationality and accounting for over ten percent of the total deaths in the world each year (World Health Organization, 2006). Cancer has been identified in most body organs and new forms continue to be characterized. In hopes of finding potential treatments, or a cure, human cancer has become widely studied and more becomes known about the disease daily. As more is understood about its causes, researchers are able to begin to develop treatments that specifically target pathways (e.g. ErbB) in which dysregulation leads to oncogenesis.

The ErbB family of Receptor Tyrosine Kinases

Protein kinases are essential to the human body because they are involved in regulating many biological functions through signaling pathways (Olayioye, 2001). There are nearly 60 different human receptor tyrosine kinases (RTKs) encoded by the human genome (Figure 1), which can be broken into 20 sub-families (Blume-Jensen and Hunter, 2001). These receptors each have different structures and roles in the body, but all are made up of an intracellular domain containing the kinase domain, a transmembrane domain, and an extracellular domain (Blume-Jensen and Hunter, 2001). Additionally, tyrosine kinase receptors are involved in many different cell signaling pathways that relay information to the nucleus (Schlessinger and Lemon, 2006). These intracellular pathways are responsible for regulating many cell functions such as proliferation, differentiation, migration, and apoptosis.

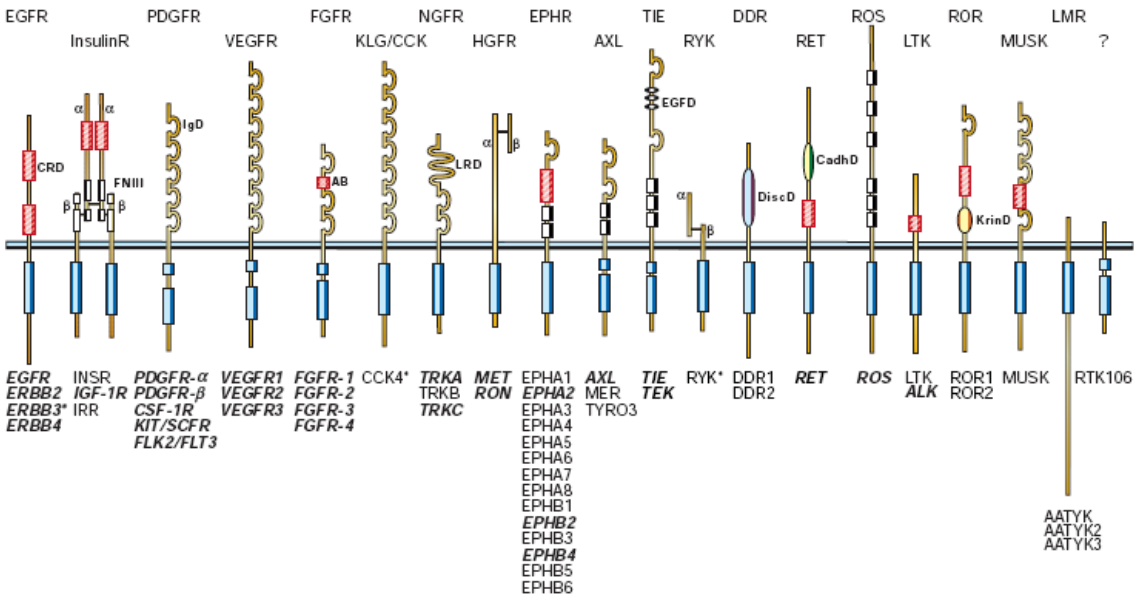

Figure 1: Human protein tyrosine kinase receptor families. All together, there are 58 different human RTKs split into 20 different families. This figure illustrates the many variations among the different RTKs. (Taken from: Blume-Jensen and Hunter, 2001)

Among the RTKs, the ErbB family is one of the better studied families of receptors. The ErbB family consists of 4 different receptors: Epidermal Growth Factor Receptor (EGFR/EGFR), ErbB2, ErbB3, and ErbB4 (Linggi and Carpenter, 2006, Bogdan and Klämbt, 2001). Like other RTKs, the ErbB receptors contain an extracellular ligand-binding region, a small transmembrane region, and an intracellular tyrosine kinase region surrounded by a juxtamembrane region and a carboxyl (C-) terminal tail (Burgess et al., 2003). The ErbB receptors have four extracellular subdomains, I – IV, with regions II and IV being cysteine-rich and regions I and III being important for ligand-binding. (Burgess et al., 2003; Linggi and Carpenter, 2006; Stein and Staros, 2000).

The current model proposes two forms for the ErbB receptor monomers relating to their activation. When in its inactive form, ErbB receptors are tethered monomers, with the extracellular region folded over on itself (Linggi and Carpenter, 2006). ErbB

receptors are activated through the binding of a ligand, such as EGF, to the receptor and the subsequent dimerization of the receptor as shown in Figure 2 (Burgess et al., 2003; Linggi and Carpenter, 2006). The model suggests that ligand binding causes the dimerization arms of the ErbB receptors to become exposed, allowing two receptors to dimerize (Lemmon et al.,1997; Burgess et al., 2003). Dimerization activates the kinase activity of the receptors which in turn causes the monomers to transphosphorylate tyrosine residues in their C-tail (Linggi and Carpenter, 2006). This intermolecular phosphorylation of C-tail tyrosines triggers signaling pathways in the cell. The nature of the pathway triggered depends on many factors, such as the ligand bound and the dimerized receptors (Yarden and Sliwkowski, 2001).

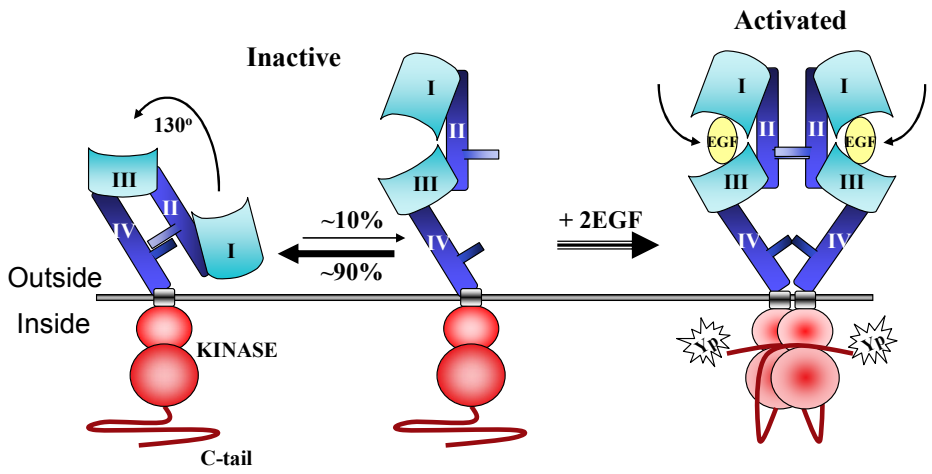

Figure 2: Activation of the ErbB receptor. In its inactive form, EGFR typically has a tethered, folded-over structure, but when EGF ligand binds, the EGFR opens and is able to dimerize with itself or another ErbB receptor. This dimerization activates the receptor and triggers autophosphorylation of the C-tail tyrosine residues.

Each ErbB receptor is thought to have a slightly different role in the body, and have different ligand specificities but only EGFR and ErbB4 are fully functional,

containing both ligand binding and kinase activity (Burgess et al., 2003). The ErbB2 receptor lacks ligand binding activity, rendering it inoperative unless it dimerizes with another ErbB family member, capable of binding to ligand (Burgess et al., 2003; Linggi and Carpenter, 2006). ErbB3, on the other hand, has a working extracellular receptor region, but an inactive tyrosine kinase domain, so it is unable to phosphorylate C-tail tyrosines, but can act as a substrate for the kinase domain of another receptor if a heterodimer is formed (Burgess et al., 2003). Although ErbB2 and ErbB3 alone lack full receptor binding and kinase functions, the ErbB2-ErbB3 heterodimer is the most mitogenic and transforming RTK complex (Yarden and Sliwkowski, 2001). Additionally, it has been suggested that EGFR is capable of bypassing canonical signaling cascades and translocating to the nucleus where it can more directly regulate gene expression and cellular processes (Lo and Hung, 2006).

ErbB Receptor Signaling Pathways and Cancer

The ErbB family has been implicated in many types of human cancer and, as a result, is one of the most studied signaling networks. Commonly, cancer occurs when the ErbB pathway becomes hyper-activated by the overproduction of ligands, overproduction of ErbB receptors, or constitutive activation of ErbB receptors (Yarden and Sliwkowski, 2001). Many experiments have shown that ErbB RTKs lead to development of cancer, particularly through misregulation of the phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways (Holbro et al., 2003; Yarden and Sliwkowski, 2001). This misregulation often occurs as a result of mutations in the ErbB family, which leads to overexpression or constitutive activation of the receptor signaling pathways (Holbro et al., 2003; Yarden and Sliwkowski, 2001).

In an ordinary cell, the autophosphorylation of ErbB receptors triggers the activation of intracellular pathways necessary for normal development (Yarden and Sliwkowski, 2001). Mouse models have shown that ErbB receptors are vital to normal development and survival. As reviewed by Hynes and Lane, mice embryos without ErbB2, which is required for development of the heart, die due to improper chamber formation and blood flow in the heart, while mice without EGFR die soon after birth, due to respiratory problems as well as skin and gastrointestinal problems (2005).

The activation of different intracellular signaling pathways as a result of ErbB RTK activation and autophosphorylation is based principally on the adaptor molecules that bind to the phosphorylated tyrosines in the ErbB receptor C-tail (Batzer et al., 1994). As shown in Figure 3, both the PI3K and MAPK signaling cascades triggered by the ErbB receptors lead to a nuclear cell signal. Phosphophotyrosine binding proteins with SH2 or PTB domains commonly act as adaptors between the activated ErbB receptor and RTK signaling pathway (Batzer et al., 1994; Luschnig et al., 2000). Two adaptor proteins, Grb2 and Shc, provide a common link between the EGFR receptor and two main signaling cascades: MAPK and PI3K (Batzer et al., 1994; Luschnig et al., 2000). It is suggested that Grb2 and Shc form a complex, together binding to specific phosphotyrosine residues on EGFR and then linking to SOS, thereby triggering the MAPK pathway. PI-3K binds directly to phosphorylated tyrosines in ErbB3 and ErbB4 via SH2 domains on its p85 subunit, but binds indirectly to EGFR through a Shc adaptor molecule, GAB1, that binds via Grb2 (Hynes and Lane, 2005; Blume-Jensen and Hunter, 2001).

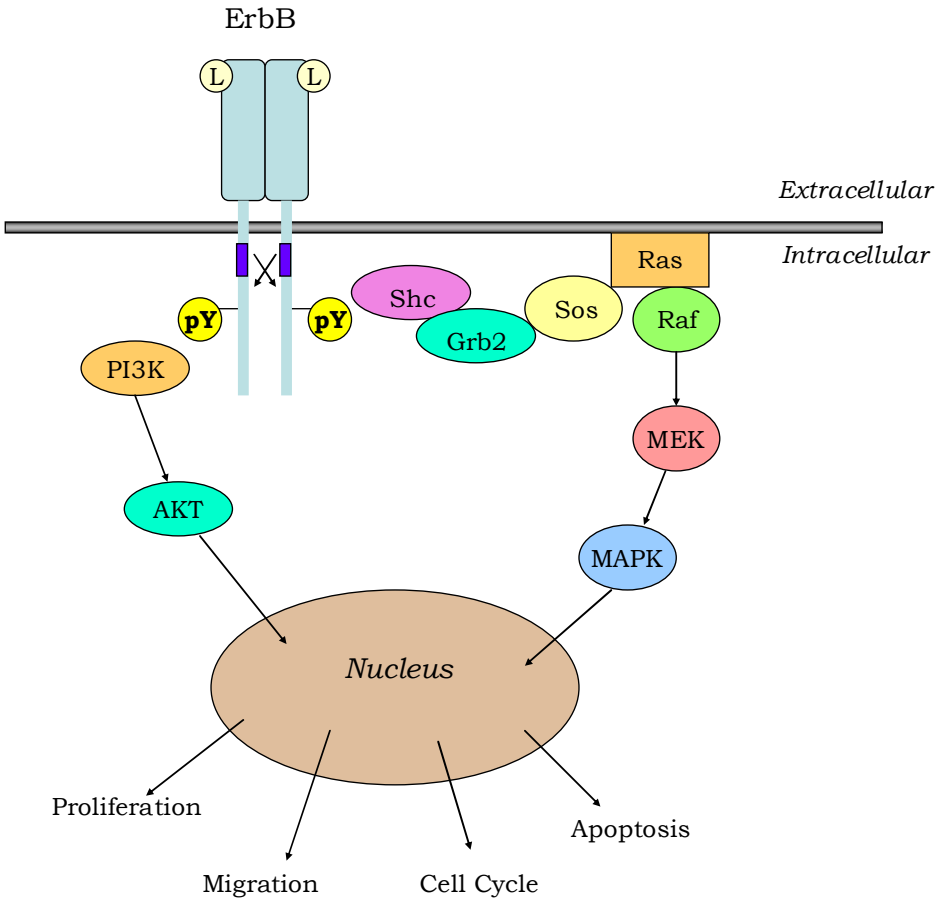

Figure 3: Oncogenic signaling pathways activated by the activation of ErbB receptors. Binding of ligand to the receptor is responsible for the cross phosphorylation of the receptor dimer and subsequent pathway activation. Conserved phosphotyrosine sequences bind to the SH2 domains of signaling molecules which activate the PI3K/AKT (left) and MAPK (right) pathways which have been implicated in many cancers.

Receptor Tyrosine Kinase (RTK) Inhibitors

As was previously discussed, RTKs function in regulating the proliferation, differentiation, migration, metabolism, and survival of the cell (Calbiochem, 2006). Since it is known that hyperactivation of these kinases in many cases leads to cancer, the use of RTK inhibitors is becoming of more interest as a novel cancer treatment. Receptor tyrosine kinase inhibitors are promising cancer therapeutics because of their ability to block the kinase activity of the receptor, inactivating the pathway. Many ErbB tyrosine

kinase inhibitors (TKIs) work by binding somewhere at the ATP pocket, which blocks ATP from binding to the kinase domain of the receptor (Baselga, 2002; Janmaat and Giaccone, 2003). The binding of these small molecule TKIs prevents signal transduction from the ErbB receptor by blocking ATP binding and kinase activity; thereby preventing autophosphorylation of the receptor (Baselga, 2002; Janmaat and Giaccone, 2003).

Development of Receptor Tyrosine Kinase Inhibitors

As more has become known about the molecular basis of cancer, molecularly targeted compounds, such as TKIs, have been developed as new cancer therapies. With the growing knowledge base, it is hoped that the design of cancer drugs will become less costly, and more effective (Benson et al., 2006). With so many different molecular compounds available, and so few proving worthwhile as cancer therapeutics, it is important to "validate" potential cancer target drugs before investing extensive time and funding into their development (Benson et al., 2006). As a cost and time saving measure, it has now become common to screen chemical and molecular libraries for promising compounds prior to any clinical trials to "validate" them as potential drugs. Recombinant catalytic-kinase domains are often used to screen for protein kinase inhibitors, and as a result, many TKIs work by competing with ATP (Sebolt-Leopold and English, 2006). This drug screening process is important because it is allows scientists to initially reject compounds that would likely produce only weak effects.

Quinazolines

Hundreds of additional TKIs have been discovered or synthesized. Of the many known TKIs, one class of inhibitors, the quinazolines, appears to be the most promising clinically for EGFR inhibition (Baselga, 2002). These compounds covalently bind to the

ATP pocket of the kinase domain of the ErbB receptors, competing with ATP binding and inhibiting the activation of the kinase domain (Discafani et al., 1999; Fry et al., 1998). As a result, they have to potential to block the EGFR pathway by inhibiting the autophosphorylation that triggers the oncogenic pathway. Two of these chemical inhibitors, CL-387,785 and PD 168393, have been shown to block activation of the EGFR pathway and are further examined as experimental cancer therapeutics in this project.

N-[4-[(3-Bromophenyl)amino]-6-quinazolinyl]-2-butynamide (CL-387,785) is an irreversible tyrosine kinase inhibitor. CL-387,785 is a quinazoline compound with chemical formula $C_{18}H_{13}BrN_4O$, comprised of a benzene ring fused with a pyrimidine as shown in Figure 4. It was developed after molecular modeling of the EGFR kinase domain, and looks promising as a clinical EGFR inhibitor for cancer treatment (Discafani et al., 1999). CL-387,785 competes with ATP binding of the receptor, which is able to significantly decrease, or inhibit altogether, the kinase domain's autophosphorylation of the receptor tail (Discafani et al., 1999). It appears to work by alkylating Cys^{773} and covalently binding with the EGFR (Discafani et al., 1999). Initial experiments showed that CL-387,785 blocked EGFR autophosphorylation and inhibited tumor growth both *in vitro* and *in vivo* (Discafani et al., 1999).

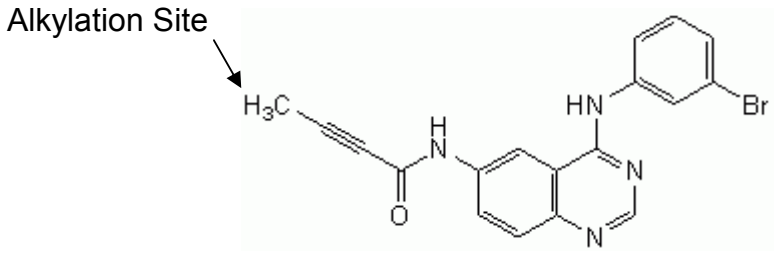

Figure 4: Chemical Structure of CL-387,785 highlighting the binding site with EGFR. Picture adapted from the EMD/Calbiochem website, http://www.emdbiosciences.com/product/233100

Additionally, 4-[(3-Bromophenyl)amino]-6-acrylamidoquinazoline (PD 168393) is an irreversible EGFR tyrosine kinase inhibitor (Fry et al., 1998). A small, quinazoline molecule with chemical formula $C_{17}H_{13}BrN_4O$ (Figure 5), PD 169393 is a strong inhibitor of the EGFR. The compound works by alkylating Cys^{773} which inhibits ATP binding and prevents activation of the EGFR kinase domain (Fry et al., 1998). With an inactive kinase domain, EGFR is unable to autophosphorylate tyrosines in its tail, and the pathway is not activated. Experimental *in vivo* data, as discussed by Fry et al., showed that PD 168393 was a strong inhibitor of EGFR and was much more potent than other known inhibitors (Fry et al., 1998).

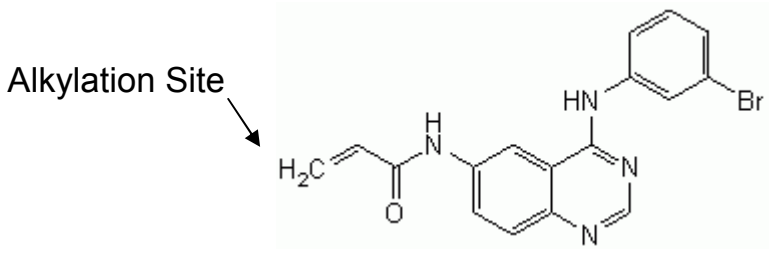

Figure 5: Chemical Structure of PD 168393 highlighting the binding site with EGFR. Picture adapted from the EMD/Calbiochem website, http://www.emdbiosciences.com/product/513033

Clinical Data

Based on some initial molecule screenings, several tyrosine kinase inhibitors have shown positive inhibition results and are already undergoing preclinical or clinical trials

as treatments for cancer (Baselga, 2002; Janmaat and Giaccone, 2003). Each TKI is slightly different, regarding its binding specificity and reversibility as an inhibitor, but several look promising as treatments once further research and clinical trials occur.

Additionally, several tyrosine kinase inhibitors targeting different members of the ErbB receptor have already been approved for use in the treatment of several types of cancers (Hynes and Lane, 2005). Two current human clinical TKIs for EGFR, Gefitinib and Erlotinib, are approved as a therapy for non-small cell lung cancer (NSCLC) once the primary and secondary treatments have proven unsuccessful (Hynes and Lane, 2005; Janmaat and Giaccone, 2003). Furthermore, these therapeutics might prove to be effective for more than NSCLC. Gefitinib, for example, is being evaluated as a therapy for head and neck squamous-cell carcinoma, gastrointestinal cancer, and breast cancer and Erlotinib is undergoing trials for the treatment of additional types of cancer as well (Hynes and Lane, 2005).

Drosophila as a Model

The utility of *Drosophila melanogaster*, commonly known as the fruit fly, as a system to model human disease and test potential treatments is rapidly increasing. Since the sequencing of both the human and *Drosophila* genome, it has been shown that a majority of human disease genes have been conserved in *Drosophila* (Fortini et al., 2000). With the finding that 62% of the human disease genes are conserved, researchers have been turning more and more to *Drosophila* to model genetic diseases (Rubin et al., 2000).

Additionally, 68% of the human cancer genes appear to have a homologous sequence in *Drosophila*, including the ErbB family of genes which correlate with the

Drosophila EGFR gene, dEGFR (Rubin et al., 2000). Although they are relatively small organisms, *Drosophila* contain many highly organized signaling pathways regulated by numerous intracellular and extracellular molecular signals, e.g. the MAPK pathway (Li and Garza, 2003). *Drosophila* are currently used for genetic screening, and it seems that with little effort flies could be used to screen compounds as well. While cellular assays are often used, they are less than ideal since they are often based solely on target interaction and can be limiting as a chemical genetics screen (Li and Garza, 2003). The drug effects can be studied in a living organism in more detail than is possible with a cultured system, paying attention to toxicity and off-target effects (Manev et al., 2003).

Additionally, the use of GAL4-UAS system in *Drosophila* allows for targeted expression of a gene of interest (Duffy, 2002). The crossing of the responder (UAS) and driver (GAL4) enables the overexpression of a gene and the corresponding phenotype (e.g. lethality) can be studied (Duffy, 2002; Li and Garza, 2003). Taking it one step further, compounds that inhibit these phenotypes could be identified by using a chemical genetics assay by observing phenotypic reversion (Li and Garza, 2003).

For such studies, *Drosophila* oogenesis provides an experimental system well suited for such chemical genetics studies. Previous studies have shown that *Drosophila* females can be fed small molecules and effects can be noted on developing egg chambers. For example, feeding females colchicine led to the disruption of microtubule structure and pattern in developing egg chambers (Townsley and Bienz, 2000). As more studies have shown the ability to feed *Drosophila* chemicals and observe phenotypic results, flies have been identified as a potential model for pharmacological research.

Additionally, methods of administering drugs via feeding, injection, or inhalation of desired chemicals, show the practicality of the *Drosophila* model (Manev et al., 2003).

In *Drosophila* dEGFR activity within the follicle cells determines the dorsal fates, and dorsal ventral polarity of the eggshell (chorion) provides a very simple marker for dEGFR activity. Specification of dorsal appendages allows the different levels of dEGFR activation to be identified, allowing for easy phenotypic characterization of activity. Wildtype flies lay chorions with 2 dorsal appendages, while flies lacking EGFR activity lay ventralized chorions. In contrast, flies with hyperactive EGFR signaling lay dorsalized chorions, easily observed by the presence of ectopic dorsal appendages.

STATEMENT OF PURPOSE

Many experiments have confirmed the relationship between misregulation of the ErbB receptors and the development of certain types of cancer. While ErbB receptors are necessary for normal development, the hyperactivation of these receptors and subsequent autophosphorylation of their kinase domains leads to the constitutive activation of downstream signaling proteins, thereby contributing to cancer. To date, the most common cancer treatment is chemotherapy, with additional molecular drugs used as a second or third option when initial treatments do not seem effective. Still, much time and money are being put into developing targeted cancer therapeutics with the hope of increased efficacy and reduced toxicity.

With the conservation of the receptor and intracellular signaling pathways between *Drosophila* and humans, it seems practical that fruit flies could be used to screen for cancer therapeutics. Additionally, with the ability to make flies transgenic for the human ErbB gene, it is possible to use *Drosophila* for chemical genetic screens to identify cancer drugs targeted specifically to the human ErbB family members. Instead of relying on a high throughput cell culture screen, an assay using *Drosophila* would enable the inhibitor effects to be studied in a complete organism instead of a cell line.

The goal of this project was to determine if *Drosophila* could be used to identify novel therapeutics for EGFR dependent cancers. Additionally, development of a high throughput chemical genetics assay using *Drosophila* oogenesis was desired to screen chemical libraries to identify inhibitors of RTKs implicated in human disease.

MATERIALS AND METHODS

Bioinformatics

Using the full length sequences for the human EGFR, ErbB2, ErbB3, and ErbB4 proteins as well as the *Drosophila* dEGFR protein, a ClustalW 1.8 sequence alignment (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html) of the intracellular regions for each protein RTK was performed. The resulting alignment was imported into the Curation and Alignment Tool for Protein Analysis (CATPA) program (http://www.catpa.org). Using CATPA, sequences were then annotated with respect to tyrosine residues, kinase domains, and sequences surrounding C-tail tyrosines that were conserved between the human and fly receptors.

Using the online UniProt Knowledgebase (http://ca.expasy.org), the kinase domain for each receptor of was identified and annotated in CATPA. To confirm the alignment and CATPA analysis, each protein was also individually screened by eye for tyrosine residues after the kinase domain. The sequences surrounding each tyrosine were compared to determine which tyrosines were conserved between the ErbB family and dEGFR. Once the conserved sequences surrounding the tyrosines were determined, it could be predicted what adaptor molecules would likely bind to the phosphorylated tyrosine residues.

Additionally, the complete sequences of other human RTKs were obtained online from both the Uniprot Knowledgebase and NCBI Entrez Protein database (www.ncbi.nlm.nih.gov/Entrez/). Using the two conserved tyrosine consensus sequences (sxLqRYsxDPt, VxNPEYL) obtained from the ErbB family analysis, the RTK family members were screened for similarities in intracellular tyrosine residues.

Drosophila Genetics

Virgin female flies from stocks [*UAS-EGFR·GFP]^{KC7a} / TM3,Sb*, [*UAS-dEGFR1·GFP]$^{1E\ III}$, [UAS-EGFR·GFP]^{KC7b} / CyO*, *[UAS-EGFR]$^{22\ II}$*, *[UAS-ErbB3]$^{24-2}$ / TM3,Sb* and *[UAS-ErbB3]^{66} / CyO* were collected and crossed with males for different drivers ([*CY2-GAL4]*, *[Act-GAL4][tub-GAL80^{ts}]^{R2} / CyO*, *[CY2-GAL4][tub-GAL80^{ts}]^{R9} / CyO*, *[GMR-GAL4][UAS-EGFR·GFP]^{KC7b} / CyO*). These flies were allowed to mate in a vial with yeast for a few days and flipped into new vial or bottle to continue mating and produce many progeny. The F1 generation was sorted by phenotype to select for the correct genotype, and female flies of the correct genotype were used for drug testing and egg lays, as well as anti-EGFR, anti-dEGFR staining, and GFP staining. Wild-type, *W^{1118}*, flies were also maintained and used for experimental controls.

Unless otherwise noted, all flies were kept on standard media at room temperature. Crosses with the temperature sensitive strains, *[Act-GAL4][tub-GAL80^{ts}]^{R2} / CyO*, and *[CY2-GAL4][tub-GAL80^{ts}]^{R9} / CyO*, were kept at 28.5°C to deactivate *GAL80*.

Apple juice Agar

Typical agar recipes were modified to include apple juice and additional sugar to attract fruit flies. For one liter of agar, 22 grams of agar was dissolved in 500 mL of distilled water in the microwave until in solution. Half a can of frozen apple juice was heated and mixed with 32 grams of sucrose and enough dH_20 to make 500 mL of solution. When both solutions were dissolved, they were combined, and poured into individual or multi-well plates. Remaining apple juice agar was covered and stored at 4˚C.

Chorion Preps

Chorions from the various *Drosophila* F1 genotypes were obtained using egg lay bottles. 8-10 female flies from each genotype of interest and 2-3 male flies were placed in empty bottles with small holes to allow in oxygen. Plates made with apple juice agar were dotted with a yeast paste and put on the bottle tops. The plate was taped on to keep the flies inside, and the bottles were turned upside down and left overnight at 25°C. The following day, the original plates were replaced with new apple juice plates, and the chorions were observed and collected from the plates (Figure 6). Chorions were placed on a slide in Lacto-Hoyer's (50-50) solution, and placed overnight on a 65°C hotplate. The chorions were viewed using dark field microscopy at 100x magnification and images were taken.

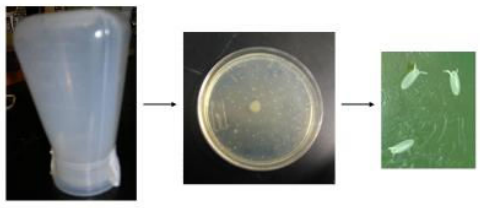

Figure 6: Example of *Drosophila* egg lay procedure. Flies were placed overnight into upside-down bottles with apple juice agar plates at the bottom, as shown on the left. The following day, the apple juice plates were removed and the chorions inspected under a dissection microscope. The egg lay plate with chorions is shown in the center image and an example of wild-type chorions viewed under the microscope is shown on the right.

Histochemistry

Between 6 and 8 flies per genotype were knocked out with CO_2 and their ovaries were removed and dissected in PBT (0.1% Tween20 in 1xPBS) in a glass dish. The dissected ovaries were fixed in 3.7% formaldehyde in PEMP (10 mL 0.5M PIPES, 100µL 1M MgSO4, 100 µL 0.5M EDTA, 50µL NP-40, 39.75 mL dH_20) for 15 minutes.

The ovaries were rinsed 3 times in the glass dish with Ab wash (20 mL 1M TrisCl pH 7.4, 6mL 5M NaCl, 200 µL NP-40, 200 mg BSA, 174 mL dH_20) before being transferred to a 0.5 mL Eppendorf tube for 2 additional rinses. The ovaries were blocked with Ab block (5% BSA (5mg/mL) in Ab wash) for 1 hr at room temperature. The block was removed, and rotated overnight at 4˚C in 50 µL 1˚ antibody (1:1000 dilution in Ab wash for mouse anti-EGFR Ab-12 cocktail (Neo Markers) and rabbit anti-dEGFR(Duffy Lab)). The 1˚ antibody was removed, and the ovaries were washed 8-10 times in Ab wash for 5 minutes each time. The 2˚ antibody (Alexa Fluor 488, specific to the 1˚) was diluted 1:400 in Ab wash and rotated for 2 hours at room temperature. Remove 2˚ antibody and wash 8-10 times in Ab wash for 5 minutes each before mounting in 70% glycerol. Once mounted on slides, the ovaries were observed using fluorescent microscopy to observe EGFR and dEGFR expression and localization.

Between 6 and 8 flies per genotype were knocked out with CO_2 and their ovaries were removed and dissected in PBT in a glass dish. The dissected ovaries were fixed in 3.7% formaldehyde in PBT for 15 minutes. The ovaries were rinsed 3 times in the glass dish with before being transferred to a 1.5 mL Eppendorf tube for 2 additional rinses. The ovaries were then mounted in 70% glycerol, mounted on slides, and observed using fluorescent microscopy to observe GFP production and localization in the cells.

Inhibitor Treatments

CL-387,785 (catalog # 233100) and PD 168393 (catalog # 513033) were ordered from Calbiochem. The stock vials of each TKI were taken up in 200ul DMSO for a concentration of approximately 13mM for each. For the *Drosophila* drug treatments,

some stock CL-387,785 (CL) and PD 168393 (PD) were diluted in a dilute apple juice dH_20 mixture to obtain various concentrations (1:100 – 1:500) for the drug treatments.

The *Drosophila* F1 generation expressing the CY2-GAL4 driver were used for inhibitor screening. For the project, it is important to maximize and optimize the drug screening capability, while maintaining sufficient phenotypic data to analyze the drug effects so different sized multi-well plates, different numbers of flies, and different drug concentrations were used to determine the optimal testing conditions. Table 1 shows the various experimental conditions tested for the assay.

Trial #	Flies per well (in 48 well plate)	Inhibitor & Concentration	Other variables
1	3 females	10 µL CL (1:500)	RT, flies >3 days old
2	3 females	10 µL CL (1:500)	Flies ~2 days old, room temp ~21°C
3	3 females	10 µL CL (1:500)	Temp in room varied, flies ~2 days old
4	3 females + 1 WT male	10 µL CL (1:500)	Flies starved ~ 6 hrs before, ~2 days old, RT
5	3 females + 1 WT male	10 µL CL (1:100) or 10 µL PD (1:100)	Dark agar, starved flies, room temp varied, removed wings, ~2 days old
6	3 females + 1 WT male	10 µL CL (1:100) or 10 µL CL (1:500) or 10 µL CL (1:1000)	25°C incubator, starved, removed wings, ~2days old

Table 1: Experimental Conditions for Chemical Genetics Assay.

RESULTS

Identification of Conserved Adaptor Binding Sites

A Curation and Alignment Tool for Protein Analysis (CATPA) data file was created for the ErbB and dEGFR protein (Figure 7). Within the alignment file, the kinase domains and conserved tyrosine residues were highlighted to show conserved sequence homologies. Additionally the program allowed comments to be added about the residue/domain of interest so experimental data could be tied with the sequence data in the same file.

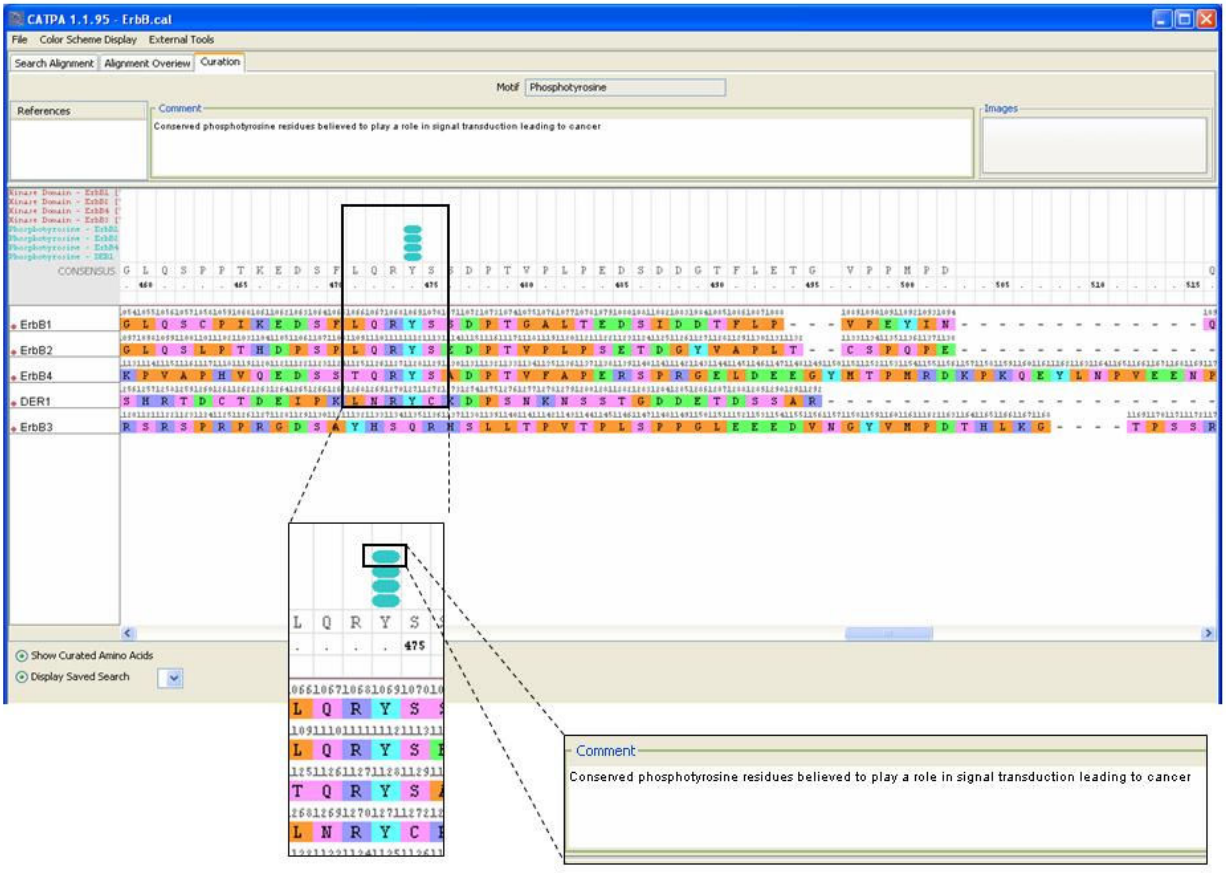

Figure 7: Screenshot of the CATPA Program. Features of the program are highlighted such as the comment feature and ability to mark off residues and/or domains. In addition to a color scheme showing conserved and similar residues, the individual conserved tyrosine sequences were marked off and labeled for easy visualization. By clicking on a labeled residue, the user is able to pull up useful information such as images from outside experimental data, creating a data file with useful information about the proteins of interest.

The goal of the sequence analyses of EGFR, ErbB2, ErbB3, ErbB4 and dEGFR was to identify conserved tyrosine residues. Since the tyrosines (Y) in the kinase domain are not essential for activation of intracellular pathways, focus was placed only on conservation of the C-tail Y residues (Table 2). Since phosphorylated tyrosines in dEGFR activate the fly MAPK pathway, it was expected that the human ErbB receptors would only activate the pathway if it contained residues that were conserved with dEGFR.

	EGFR	ErbB2	ErbB3	ErbB4	dEGFR
Tyrosines in tail	9	9	14	19	14
Tyrosines Conserved	2	2	0	1	2

Table 2: C-tail tyrosine analysis data for ErbB and dEGFR.

Two tyrosine sequence homologies between the human and *Drosophila* receptor tail were identified (Table 3). The two consensus sequences were identified, sxLqRYsxDPT and VxNPEYL. The first, sxLqRYsxDPT, is conserved within EGFR, ErbB2, ErbB4, and dEGFR, while the second, VxNPEYL, is conserved in EGFR, ErbB2, and dEGFR.

Consensus	**SPxDSxFYRxL**	**vDAeEYLvPQqgFf**	**sxLqRYsxDPt**	**PeYhN**	**VxNPEYL**	**tAENpEYL**
EGFR	SPTDSNF**Y**RAL (Y-998)	VDADE**Y**LIPQQGFF (Y-1016)	SFLQR**Y**SSDPT (Y-1069)	PV**Y**HN (Y-1110)	VGNPE**Y**L (Y-1138)	TAENAE**Y**L (Y-1197)
ErbB2	SPLDSTF**Y**RSL (Y-1005)	VDAEE**Y**LVPQQGFF (Y-1023)	SPLQR**Y**SEDPT (Y-1112)	PE**Y**VN (Y-1139)	VENPE**Y**L (Y-1196)	TAENPE**Y**L (Y-1248)
ErbB3						
ErbB4		MDAEE**Y**LVPQAFN (Y-1021)	SSTQR**Y**SADPT (Y-1128)	PE**Y**HN (Y-1188)		VAENPE**Y**L (Y-1284)
dEGFR			PKLNR**Y**CKDPS (Y-1271)		VDNPE**Y**L (Y-1357)	

Table 3: Consensus tyrosine sequences between the ErbB family and dEGFR. For each receptor, residues surrounding the tyrosine residues in the C-tail were compared to determine sequence similarities and a consensus sequence for the receptor family. Capital letters in the consensus sequence indicate residues conserved in all receptors, lowercase letters indicate conservation in most, and x indicates a lack of consensus for that residue. The number of each receptor's tyrosine residue for each consensus sequence is listed in parentheses.

Furthermore, it was determined to which signaling pathway molecules the conserved tyrosines might bind, focusing only on the two sequences that were conserved between ErbB and dEGFR (Figure 8). By focusing on the conserved sequence and tyrosine residue number for each receptor, adaptor molecule(s) that are predicted to interact with the ErbB/dEGFR conserved phosphotyrosine residues were identified. The consensus sequences VxNPEYL, found surrounding EGFR (Y-1138), ErbB2 (Y-1196), and dEGFR (Y-1357), is predicted to bind to a Shc-Grb2 complex through the interaction of the Shc PTB domain with the receptor phosphotyrosine residue (Batzer et al., 1995; Olayioye, 2001; Schultze et al., 2005). The binding of this complex to the activated receptor triggers the MAPK pathway (Seet et al., 2006). Additionally, the consensus sequence sxLqRYsxDPT, shown to be conserved between EGFR (Y-1069), ErbB2 (Y-1112), ErbB4 (Y-1128), and dEGFR (Y-1271) is predicted to bind to the SH2 domain of Grb2 (Batzer et al., 1994; Olayioye, 2001; Schultze et al., 2005). Grb2 binding, as previously discussed, is responsible for activating the MAPK pathway (Seet et al., 2006).

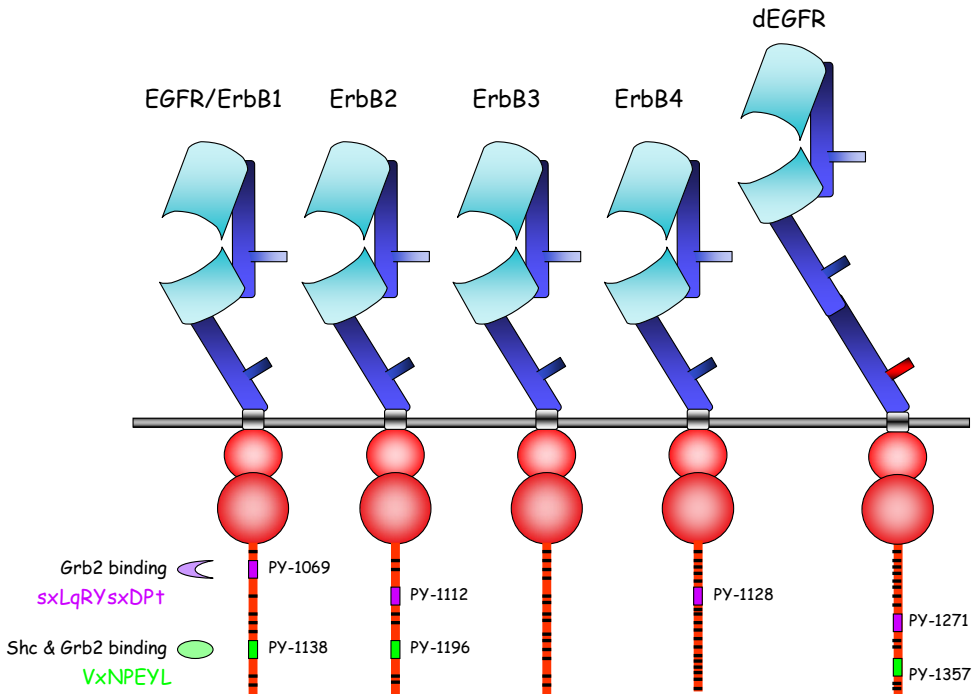

Figure 8: The tyrosine residues on the ErbB and dEGFR tails. The first set of conserved sequences, shown in purple, appears responsible for the binding to a SH2 domain, such as is found in Grb2 protein. The second set, shown in green, appears responsible for the binding of a PTB domain, such as is found in Shc. The colored, boxed regions on the tail show conserved tyrosine residues and their location in the receptor sequence. The related consensus sequences and the potential molecules that bind to the phosphorylated tyrosines are shown on the left side of the figure. The dashes in the tail show the tyrosine residues that were not conserved.

After the analysis of the ErbB receptor family, it was decided to screen selective members of the remaining RTK families to determine if any experimental data could be applied beyond the ErbB RTKs. Receptor sequences were obtained and screened for the dEGFR and ErbB consensus residues, sxLqRYsxDPT and VxNPEYL. Analysis of those sequences showed 7 out of 18 receptors with at least partial conservation (Table 4) of the residues in the ErbB and dEGFR consensus.

Receptor Name	Accession number	Conserved Y residues
PDGFRb	NP_002600	
INSR	AAA59452	NPEYL (Y-999)
VEGFR	NP_002010	PEY (Y-1124)
FGFR1	AAH15035	NqEYL (Y-764)
TRKA	BAA34355	PvYL (Y-791)
EPHA1	P21709	
EPHB2	P29323	
AXL	NP_068713	EYL (Y-662)
TIE	NP_005415	
TEK	Q02763 (UniProt)	
RYK	NP_001005861	
DDR1	AAH70070	
RET	AAH03072	
ROS	P08922(UnitProt)	
LTK	AAH45607	
ALK	NP_004295	PEYkL (Y-1078)
ROR1	Q01973 (UniProt)	EYL (Y-605)
MUSK	AAI09100	

Table 4: Potential Adaptor Binding Sites in other RTKs. Using the tyrosine residues conserved between dEGFR and ErbB (Table 3), other RTK family members were screened for the consensus sequence to determine if they were capable of activating the same pathways.

Functional Analysis of Human EGFR in Drosophila

Our bioinformatics analysis predicts that EGFR, ErbB2, and ErbB4, but not ErbB3 might be capable of activating the *Drosophila* MAPK pathway through association with adaptor proteins. To test this, EGFR and ErbB3 were misexpressed in transgenic *Drosophila* during oogenesis and eye development.

EGFR, ErbB3, EGFR·GFP, and dEGFR·GFP were misexpressed in the ovaries and eyes. The phenotypes were evaluated to determine which lines should be used for the development of the chemical genetics assay. Phenotypic differences were easiest to detect with CY2-GAL4 misexpression so they were pursued for use in the development of the assay. The wildtype, EGFR and ErbB3 chorions appeared normal, while the chorions from EGFR·GFP and dEGFR·GFP genotypes showed dorsalization effects, demonstrating that these transgenes were capable of hyperactivating the EGFR pathway (Figure 9).

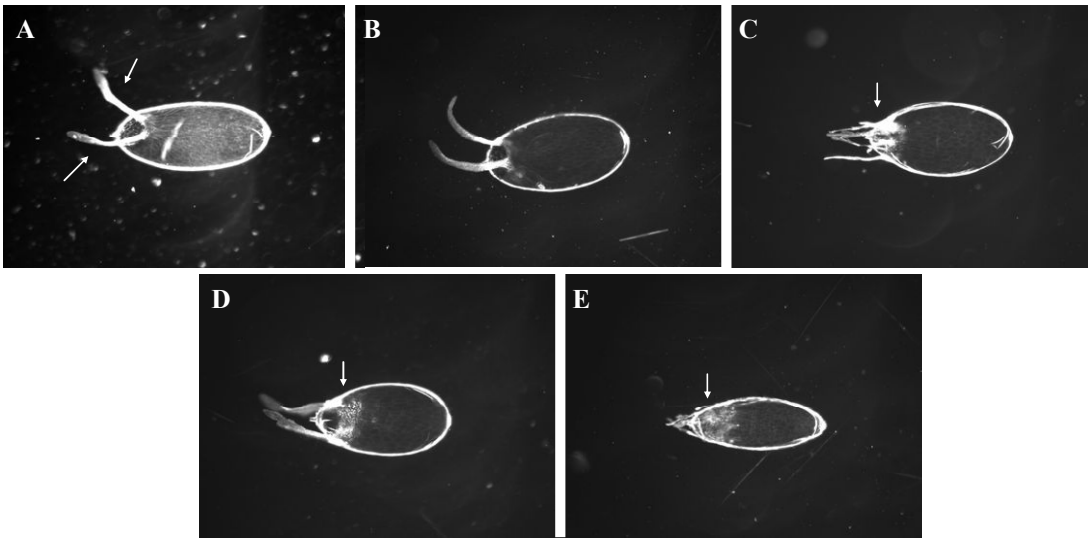

Figure 9: Chorion images from egg lays. Chorions from flies misexpressing the noted transgene under the control of the CY2-GAL4 driver (A) wildtype W^{1118} , (B) EGFR, (C) dEGFR, (D) EGFR·GFP^{KC7a} , and (E)EGFR·GFP^{KC7b} flies were observed at 100x magnification using dark field microscopy. The arrows indicate the appendage material on the chorions. Dorsalization (the appearance of ectopic dorsal appendage material) of the chorion can be seen in Panels C through E, while Panels A & B appear normal with two thin appendages and little operculum material.

It was surprising that effects were only seen with EGFR·GFP and not EGFR although both contain the same conserved adaptor binding sites. Thus, to determine if the absence of effects with untagged EGFR was due to lack of expression or aberrant localization, EGFR·GFP^{KC7a}, EGFR·GFP^{KC7b}, EGFR, dEGFR·GFP, and wildtype ovaries were stained with an antibody towards either dEGFR or EGFR (Figure 10) and for GFP tagged molecules GFP expression was also assessed (Figure 11). The antibody staining for EGFR·GFP^{KC7b} ovaries was the strongest, correlating with the stronger dorsalization observed in the phenotypic egg lay data. Expression of GFP was minimally visible in dEGFR·GFP ovaries, suggesting that the dEGFR·GFP was targeted for Cbl degradation, while the human EGFR·GFP molecules may not have been degraded because the Cbl pathway is specific to *Drosophila*.

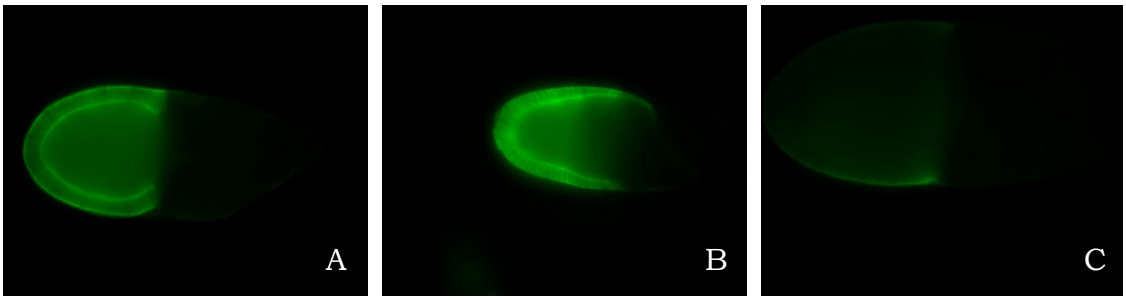

Figure 10: Anti-EGFR ovarian staining. Antibody staining of A) EGFR·GFP^{KC7a}, B) EGFR·GFP ^{KC7b}, and C) EGFR using fluorescent microscopy at 100x magnification. Note: The GFP tagged molecules showed much higher EGFR expression, suggesting a reason for the dorsalization phenotypes observed.

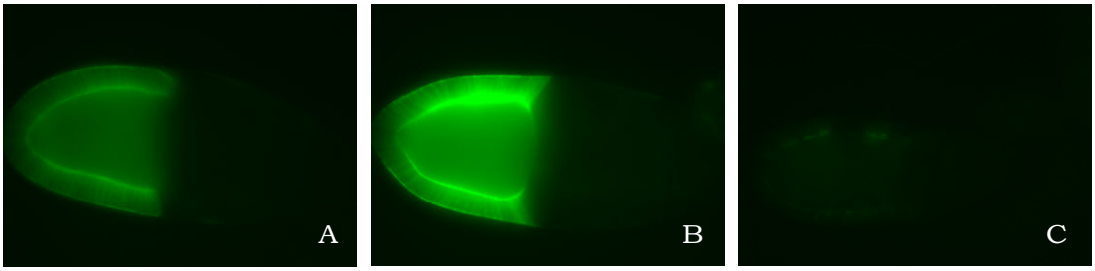

Figure 11: GFP expression in the ovaries. A) EGFR·GFP^{KC7a}, B) EGFR·GFP ^{KC7b}, and C) dEGFR·GFP were assessed for GFP expression using fluorescent microscopy at 100x magnification. Expression of dEGFR·GFP was minimal, suggesting that it may have been targeted by Cbl degradation. It was noted that, the human EGFR·GFP did not appear to be affected, showing GFP expression, suggesting that it was not subject to the same degradation.

Optimization of Chemical Genetics Assay Conditions

The ability of EGFR·GFP and dEGFR·GFP to induce dorsalization, provided an assay to examine the possibility of chemical genetics screens for the identification of TKIs. In brief, flies misexpressing these transgenes lay dorsalized chorions but feeding these females an EGFR specific TKI would lead to reversion of the phenotype to wildtype. To test this, flies were fed differing concentrations of either PD 168393 or CL-387,783 over several days and chorion phenotypes were assessed. In one trial, reversion of the chorion dorsalization was observed. To optimize the assay, adjustments to the temperature, number of flies per well, well size, drug concentration, and other variables were performed over several trials (Table 1). This preliminary reversion was unable to

be replicated. Problems encountered with the assay included: the flies stopped laying eggs after 3 or 4 days, temperature fluctuations in the room affected the level of misexpression and phenotypes, and it was difficult to assess if flies were consuming TKI and how much.

DISCUSSION

The identification of residues, surrounding C-tail tyrosines, conserved between *Drosophila* dEGFR and human ErbB highlighted the possibility of utilizing *Drosophila* for the identification of novel cancer therapeutics. The conserved sequences appear to be binding sites for pathway adaptor molecules that activate intracellular pathways (e.g. MAPK). With conserved tail sequences, sxLqRYsxDPT (conserved in EGFR, ErbB2, ErbB4 and dEGFR) and VxNPEYL (conserved in EGFR, ErbB2, ErbB4 and dEGFR) and adaptor molecules, it was likely that human ErbB receptors would to activate the fly MAPK pathway. Confirming this, experimentally *EGFR·GFP* misexpression in *Drosophila* led to dorsalization of the chorions. Since *EGFR·GFP / CY2-GAL4* is properly expressed in the cells and active, I the human receptor must be capable of associating with *Drosophila* adaptor proteins necessary for activating the MAPK pathway.

The reversion of dorsalized chorions to wildtype-looking chorions in one of the inhibitor trials indicates that the assay is feasible. Unfortunately, positive results were only seen in one of seven trials, indicating that optimization is essential before libraries of chemical compounds can be screened. Results from flies treated with known inhibitors must be able to produce suppress dorsalization in EGFR·GFP chorions at a frequency (estimated >30%) capable of being detected in a high throughput format. Assay results from treatment with known EGFR inhibitors, such as PD 168393 and CL-387,785, must be consistent before the assay can become a practical tool for library screening. Once the assay is optimized, I suggest it will be a useful tool for *in vivo* screening of compound libraries for the development of novel cancer therapeutics. With work, the chemical

genetics assay can be developed into a high throughput (384 well plate) pharmaceutical screening method.

Additionally, the assay could be expanded to screen for inhibitors for other RTKs. Since some RTK family members contained consensus sequences in their C-tail that matched the ErbB and dEGFR adaptor binding sites, it seems likely that other human RTKs expressed in *Drosophila* would activate the MAPK pathway and thus the chorion assay could be used to screen for additional receptor specific inhibitors. For RTKs without the conserved adaptor binding site, its addition could potentially enable the same assay to be used.

Together this work demonstrates this *Drosophila* assay has the potential to be a useful chemical genetics screening tool for TKIs and facilitate the development of novel therapeutics targeting a class of receptors implicated in a host of human diseases.

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